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GENE PROBES AS A TOOL FOR THE DETECTION OF SPECIFIC GENOMES IN THE ENVIRONMENT

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Gene probes hold a great promise as a tool in environmental sciences. They may be used to detect specific genotypes, to follow gene flow process, to delineate complex taxonomic aggregates and to monitor genetically engineered organisms in the environment. The sensitivity of the method is currently limited by experimental procedures and its specificity depends on the nature of the DNA sequences used as probes and the efficacy of lysing methods. Variable genetic determinants which code for the same trait determine the universality of gene probes. Finally, the method is highly feasible in terms of cost, speed and expertise. Current and future developments in molecular microbial ecology are likely to contribute toward the improvement of the probing methodology for the full realization of its potential in environmental sciences.

ABSTRACT: Gene probes hold a great promise as a tool in environmental sciences. They may be used to detect specific genotypes, to follow gene flow process, to delineate complex taxonomic aggregates and to monitor genetically engineered organisms in the environment. The sensitivity of the method is currently limited by experimental procedures and its specificity depends on the nature of the DNA sequences used as probes and the efficacy of lysing methods. Variable genetic determinants which code for the same trait determine the universality of gene probes. Finally, the method is highly feasible in terms of cost, speed and expertise. Current and future developments in molecular microbial ecology are likely to contribute toward the improvement of the probing methodology for the full realization of its potential in environmental sciences.

KEY WORDS: Gene probes; detection, sensitivity, specificity, universality, feasibility.

INTRODUCTION

Gene probes in environmental studies, what for?

Recent advances in molecular biology have resulted in a powerful methodology for the identification of specific DNA sequences. The method is based on the propensity of two complimentary denatured DNA strands to reanneal by forming base pairs. If one such strand (i.e., the probe) is radioactively or otherwise labeled, the identity of a strand of an unknown origin (i.e., target) is revealed by virtue of its ability to bind (i.e., hybridize) to the labeled probe (Meinkoth and Wahl, 1984). This principle has served extensively in the cloning of specific eukaryotic and prokaryotic DNA sequences and more recently was applied to the detection of microbial contaminants of food products (Hill et al., 1983; Fitts et al., 1983) and of drinking water (Echeverria et al., 1982). This paper discusses the potential of gene probes to become a tool in basic, as well as applied, microbial ecology.

There are several areas of research in microbial ecology that could greatly benefit by detecting specific organisms through the homology of their DNA and RNA with known gene probes.

i. Detecting functional groups of microorganisms. The phenotypic characterization of organisms freshly isolated from the environment is important to define the microbial metabolic potential of that environment. Traditionally, this has been carried out by using selective growth media, whereby organisms were detected by their growth on specific substrates or in the presence of inhibitors or by their ability to carry-out specific reactions resulting in a color change. These methods suffer from a high degree of false positive and negative results due to cross-feeding between organisms and the limited opportuni for growth provided by any laboratory

growth medium, respectively. This has been a major hindrance in studying organisms which could carry out the biodegradation of xenobiotic compounds (Sayler et al., 1985). Gene probes constructed from the DNA sequences coding for the function of interest clearly distinguish potentially active organisms by the presence of the specific gene systems in their genomes. Such DNA probes have been constructed for the detection of toluene and naphthalene (Sayler et al., 1985), 2,4-dichlorophenoxyacetic acid (Amy et al., 1985) and 4-dichlorobiphenyl (Pettigrew and Sayler, 1986) biodegradative microorganisms. In addition, organisms, whose cultivation is laborious and whose biochemical characteristics are not well defined may be distinguished by species-specific DNA probes. An example of this case was recently presented by Kuritza et al. (1986) who differentially detected species of Bacteroides in order to study polysaccharide degradation in the human colon.

ii. Gene flow in natural microbial communities. The processes and mechanisms of evolution of specific microbial activities and how they are influenced by the changing environment is a basic field of study in microbial ecology. So far, these studies have been based on the evolving microbial phenotypes. Gene probes provide a tool to look at the genetic level of the underlying molecular events of evolution in the natural environment. It was with this idea in mind that a DNA gene probe was developed for the bacterial mercury resistance gene system (Barkay et al., 1985). However, the biodegradative gene probes mentioned above could be utilized to follow gene flow in impacted environments. That approach has been taken by Sayler et al. (1985), who showed a positive correlation between synthetic oil treatment of microcosm sediments and the distribution of the TOL plasmid in the indigenous microbial community.

iii. The detection of genetically engineered organisms. The prospect of utilizing biotechnological approaches to improve the management of ecosystems presents a serious question regarding the safety of releasing GEMs into the environment. In order to evaluate their effects, the organisms must be detected in complex environmental samples and be differentiated from abundant indigenous flora. This could be achieved by using gene probes specific to the genome of the introduced species. Although, some use of this approach has already been documented (Sayler et al., 1986), its applicability remains to be evaluated.

iv. The detection of nonculturable organisms. The majority of the microorganisms in the natural environment cannot be cultured on conventional laboratory media. Some of these are fastidious microorganisms whose growth requirements have not been defined and, in addition, even culturable organisms enter unculturable life stages in response to harsh environments (Xu et al., 1982). Gene probes provide a means by which unculturable organisms can be detected by hybridization with total microbial DNA isolated from environmental samples (Sayler and Stacey, 1986). Low recovery of microbial DNA from environmental samples is a major stumbling block in the application of this approach in microbial ecology and several laboratories are engaged currently in research to improve available procedures of DNA isolation and purification (Ogram et al., 1986; Steffan et al., 1986).

Any monitoring method has to fulfill several criteria in order to be successful. A method has to be specific, reproducible, sensitive, universal and feasible in laboratories with diverse expertise and resources. The following is a discussion of these criteria in regard to gene probe hybridization as a method for environmental monitoring.

SPECIFICITY

The reliable use of a gene probe requires that the labeled sequence is homologous enough to hybridize to the detected target DNA, and different enough from unrelated sequences to avoid false negative and false positive results, respectively. Genes coding for the same function in different organisms may be heterologous if they have evolved independently. For example, the mer gene probe prepared by Barkay et al. (1985) from Shiegella flexneri, hybridized with mercury-resistant Gram-negative organisms, but not with Gram-positive ones, although in both groups resistance was mediated by the same mechanism. On the other hand, as new gene functions evolve by gene duplication and mutation, related sequences may code for completely different functions (Ohno, 1984). In addition, repeated DNA sequences (i.e., insertion elements, transposons) may result in false positive hybridization signals (Sayler et al., 1985). This problem was evaluated by hybridization of a common cloning vector, pACYC184 with a collection of Gram-negative enteric organisms, and approximately 35% of the 108 strains tested were hybridization positive, although none was resistant to chloramphenicol and tetracycline (the resistances which are coded by pACYC184)(Barkay, unpublished results). Low specificity of gene probes may lead to high background hybridization in environmental studies. This was demonstrated by a study of groundwater aquifer microcosms in which five gene probes were utilized to follow four introduced bacterial strains (Sayler et al., 1986). The specificity of the probes ranged (in increased order) from that of a whole genomic probe to detect strain ABS10 through a cloned chromosomal fragment to detect strain AHS26, to a cryptic plasmid probe for strain AOS23, and to a cloned restriction fragment of the TOL plasmid for the detection of Pseudomonas putida

(TOL$^+$). The latter strain also contained the plasmid RK2, which was detected by hydridization with a whole RK2 probe. The results (Table 1), which summarize eight microcosm samplings over an 8-week period, indicate that whereas an increase in the abundance of hybridization-positive target colonies occurred with all probes (inoculated vs natural microcosms), the background hybridization related to the degree of the probe specificity (natural microcosms). Thus, high background hybridizations were observed for RK2, ABS10, AHS24, AOS23, while TOL$^+$ colonies were found only in inoculated microcosms (Table 1).

There are several means by which the specificity of gene probes can be improved for environmental monitoring:

i) Elimination of unrelated sequences, if the molecular details of the target genes are available, can produce highly specific DNA probes (Barkay et al., 1985).

ii) The stringency under which the hybrids are formed can be increased (i.e., more homology between the probe and the target sequence is needed for hybridization to occur) to further specificity (Meinkoth and Ward, 1984).

iii) Whole genomic libraries can be screened to select those cloned fragments which hybridize only with the desired target organisms (Fitts et al., 1983; Kuritza et al., 1986).

SENSITIVITY

The appliction of gene probes to environmental studies requires high sensitivity since specific organisms are to be detected in physically as well as biologically complex and heterogeneous matrices. False negatives may result if target organisms are masked on isolation media by the growth of heterologous aerobic heterotrophs or if they fail to lyse (and thus, hybridize) by the conventional lysis procedures which were designed

for the manipulation of Escherichia coli. The latter is especially important if one tries to follow gene flow in natural microbial communities where the objective is to detect specific genes in heterologous host background. Detection of specific organisms in a community genome isolated directly from the environment may be rendered insensitive due to low recoveries or the physical interference of natural substances (e.g., clay particles, humic material (Ogram et al., 1986; Steffan et al., 1986). Thus, the limit of detection of each gene probe should be defined before experimental results can be interpreted.

Table 2 summarizes the sensitivity levels of several DNA gene probes. The variability in the methods which were employed by the different researchers exclude valid comparisons between observed limits of detection. Sayler et al. (1985) could detect colonies of P. putida carrying the TOL plasmid among 5×10^6 heterologous colonies on nitrocellulose filters. However, additional data indicated that at the high density of colonies required to achieve such high sensitivity, the number of target organisms would be underestimated due to masking. The effect of masking on sensitivity was also observed by Hill et al. (1983), whose efficiency of detection of virulent Y. enterocolitica among nonvirulent strains declined sharply when their ratio dropped below 1:200. A species specific DNA probe detected B. vulgatus only if target DNA comprised at least 2% of the DNA isolated directly from fecal material. With this sensitivity, only the 10 to 12 major organisms in feces where numbers reach 10^{10} organisms/gm, could be detected (Kuritza and Saylers, 1985). This is a rather high limit of detection and may render this methodology impractical for samples with lower population densities. Sensitivity may be improved by concentrating large volumes of samples and by growing colonies under selective conditions

which eliminate many of the heterologous organisms. Echeverria et al. (1982) and Fitts et al. (1983) achieved sensitivities of one target organism/ml of drinking waters and 4.3 organisms/ml broth respectively, by employing these approaches (Table 2). In the latter study, the filtered organisms were lysed and hybridized without growth into colonies (Fitts et al., 1983) a strategy that could be used to overcome masking by overgrown heterologous colonies.

The susceptibility of the test organisms to lysis conditions could be verified by employing a universal probe, a DNA sequence which is commonly found in all bacteria. Hybridizing the same sample with both the universal and the specific probe would eliminate false negative results due to inappropriate lysis conditions. Lysis conditions can then be manipulated to ensure optimal results with the tested sample. That inappropriate lysis was responsible for false negative results became apparent during a study which employed the mer gene probe (Barkay et al., 1985) to follow the distribution of the mercury-resistance genes in natural microbial communities. Consequently, alternative lysis strategies were designed and the results were monitored by a ribosomal DNA gene probe. Eight bacterial strains representing a wide variety of Gram-negative organisms were grown on nitrocellulose filters and lysed by the conventional method of Grunstein and Hogness (1975). Another set of colonies was treated by a modification of this method which included the sequential treatments of colonies on the filter with sucrose lysosyme solution (1.2 µg/mℓ lysozyme; 25% sucrose, 0.05 M Tris; 0.001 M EDTA; pH 8.0), 0.25 M EDTA and an SDS high pH solution (3% SDS, 0.01M Tris, 0.1 M NaCl; 0.01 M EDTA adjusted to pH 12.6 with NaOH) prior to the NaOH treatment accounted for by the Grunstein-Hogness procedure. Colonies treated by

both lysis procedures were hybridized with the ribosomal DNA probe under stringency which allowed hybridization of all the tested strains (as had been previously determined by hybridizing dot blots of clear DNA lysates with the same probe). The results (Fig. 1) indicated that whereas colonies of most of the strains were lysed by both methods, the lysozyme-SDS procedure resulted in clearer and more defined signals. Reduced smearing was due probably to the SDS treatment (Maniatis et al., 1982) included in the modification, but poor lysis could also account for the weak signals observed with the Grunstein-Hogness procedure. This is especially true with Acinetobacter calcoaceticus, Moraxella phenylpyruvica and Pseudomonas maltophilia. This observation was reproducible and thus, it appears that this method is not appropriate for the preparation of colonies of these strains for hybridization. However, the lysozyme-SDS procedure failed with a colony of Flavobacterium odoratum, as is indicated by its weak hybridization signal (Fig. 1). Obviously, in this case, the Grunstein-Hogness method provided better results. Because no one lysis procedure was found to be completely reliable, the DNA gene probe is routinely used to control for false negative results in studies employing the mer probe.

UNIVERSALITY

A reliable gene probe is one which detects all the organisms it is designed to detect, regardless of their origin or physiological condition. Whereas this is of no consequence for gene probes designed to detect a specific gene in a specific organism (e.g., detection of GEMs) (Kuritza et al., 1986, Fitts et al., 1983), the universality of probes for specific genotypes (Sayler et al., 1985) or gene systems (Barkay et al., 1985) in mixed microbial communities needs to be assessed because several alleles

may code for a similar phenotype. For example, at least five different determinants code for tetracycline resistance (Marshall et al., 1983) and at least two determinants code for arsenite/arsenate resistance (Mobley et al., 1984) in enterobacteria. The Universality of the mer probe was tested by hybridization to Gram negative mercury-resistant bacteria isolated from fecal material, soils and sediments (Table 3). Between 32 and 62 percent of the organisms did not hybridize to the mer probe, suggesting that there is at least one alternative mercury resistance determinant in nature.

REPRODUCIBILITY

The reproducibility of hybridization procedures is excellent as long as conditions are maintained stably. However, using them to monitor organisms in the environment is subjected to all the considerations pertinent to environmental monitoring protocols, in general. As this is not influenced by the probing methodology itself, this topic is not discussed further here.

FEASIBILITY

The attractiveness of gene probes as a method for routine monitoring is in its relative simplicity and the rapidity by which the results become available. Very small amounts of the probe sequences (<1 μg/reaction) are needed and results are obtained within 24 hrs. The required equipment and supplies are simple and inexpensive and an experienced lab technician can be trained and qualified to carry-out the procedure in a matter of days. The current major drawback of the method is the use of radioactive label, which is short lived, hazardous and requires special disposal arrangements. Alternative labelling approaches are now available. Biotinylated, fluorescent, and enzyme-linked probes have all been suggested

as a replacement of ^{32}P. The applicability, and especially sensitivity, of these methods require improvements before they can be used widely and reliably (Meinkoth and Wahl, 1984).

CONCLUSIONS AND FUTURE DEVELOPMENTS

Gene probes have the potential of becoming a powerful tool in monitoring specific organisms and genes in environmental samples. This method could broaden the scope of microbial ecology by answering fundamental questions whose investigation has been limited by the availability of appropriate methodologies and a cost-effective, simple tool would be provided for monitoring the distribution and abundance of specific organisms. The latter is especially significant in light of the pressing concern with the fate and effects of GEMs in the environment. The full utilization of gene probes is currently limited by technical difficulties pertaining to their sensitivity, specificity and universality. However, new developments in molecular microbial ecology show promise of improvement. Probing specific DNA sequences directly isolated from the environment, the inclusion of concentration steps and the enrichment of specific sequences by affinity chromatography (R. Colwell, personal communication) would increase sensitivity. The careful preparation of specific probes which cover all possible genetic determinants is made possible through molecular genetics. This would improve the specificity and universality of the method. For example, subgenomic DNA probes for the careful detection of <u>mer</u> sequences in natural communities are now available (A.O. Summers, personal communication). The direct application of probes to environmental samples by using autoradiography may become possible through a better understanding of the fate of naked DNA in the environment. Such a methodology would reduce the excessive sample handling which currently complicates the statistical analysis of the observations.

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Table 1. Detection by DNA:DNA colony hybridization of introduced organisms in groundwater aquifer microcosms.

TREATMENT	POSITIVE TARGET COLONIES (FRACTION OF TOTAL CFU)				
	TOL	RK2	ABS10	AHS24	AOS23
Natural[a] Sample	.001[b]	.023	.130	.010	.025
Inoculated	.066	.070	.050	.028	.126

[a] Natural aquifer material supplemented with ca. 1×10^6 cell/gm killed ABS10 strain.

[b] .001 arbitrary limit of detection.

Table 2. Sensitivity of DNA gene probes for the detection of specific organisms in environmental samples.

Probe	Test Method	Limit of detection	Reference
TOL plasmid	Colonies grown on nitrocellulose filters	1 target/10^6 colonies	Sayler et al. 1985
Bacteroides vulgatus	Whole DNA fraction from feces	Target 2% of total population	Kuritza and Salyers, 1985
Yersinia enterocolitica	Colonies grown on nitrocellulose filters	1 target/200 colonies	Hill et al., 1983
Enterotoxigenic *Escherichia coli*	Colonies grown on McConkey agar	1/ml ($1/10^5$ heterologous organisms)	Echeverria et al., 1982
Salmonella sp.	Bacterial cells on nitrocellulose filters	4.3/ml growth medium	Fitts et al., 1983

Table 3. Hybridization of the *mer* probe with DNA of mercury-resistant bacteria isolated from variable origins.

Origin	No. of strains tested	mer +1	%
Fecal material			
Urban dwellers	26	23	88
Hospital patients	42	26	62
Rural dwellers	20	9	45
Miscellaneous[2]	7	7	100
Total	95	65	68
Soil	34	23	68
Lake sediments	21	10	48

[1] Positive hybridization.

[2] Included strains whose source was not known.

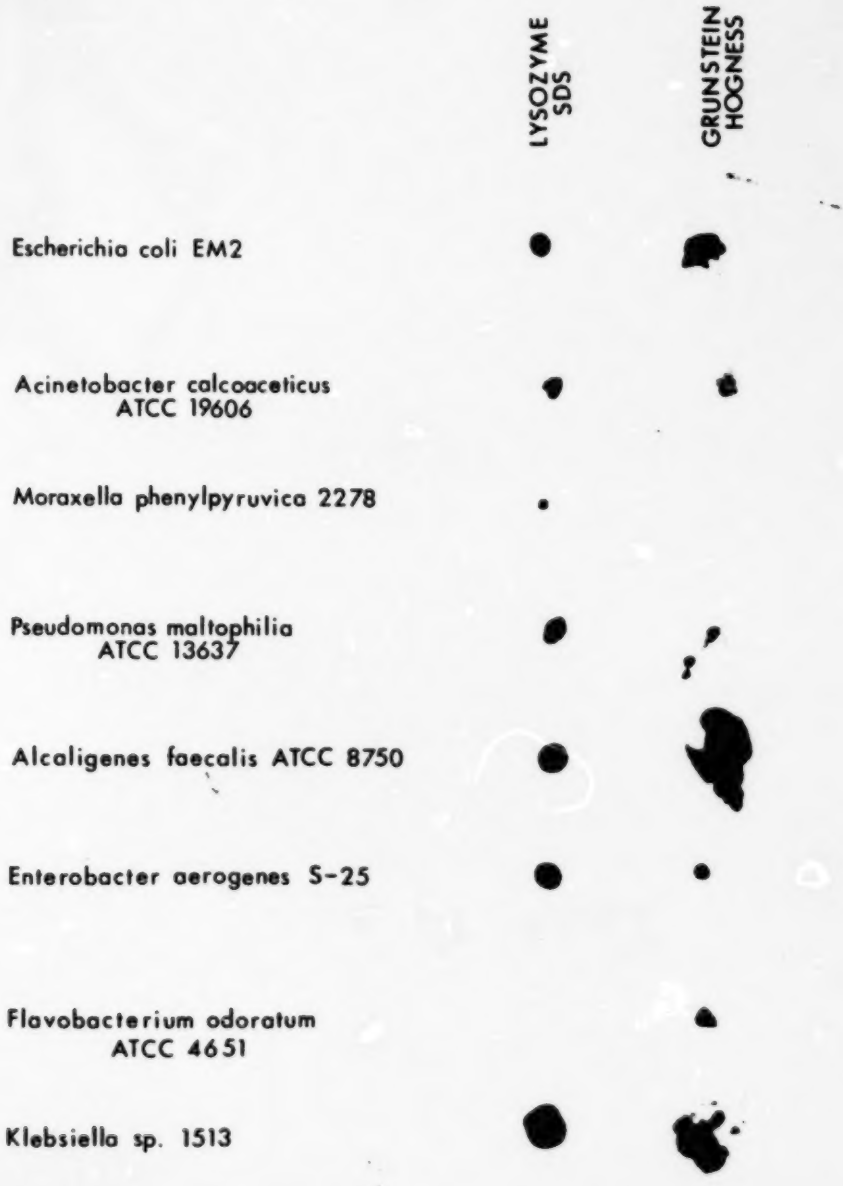

Figure 1. Colony hybridization of Gram negative bacterial strains obtained with the lysozyme-SDS and the Grunstein Hogness procedures with a ribosomal DNA probe. Colonies were lysed as described in the text (lysozyme-SDS procedure) and by Maniatis et al. (1982) (Grunstein Hogness procedure). DNA was labeled with ^{32}P-dCTP to a specific activity of 4830 Ci/umole using a nick translation kit (BRL, Bethesda, MD). The autoradiogram was obtained with XRP-5 X-ray film (Eastman Kodak Co., Rochester, NY) after overnight exposure at -70^{o}C.